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(54) Title: ELECTROPHORESIS APPARATUS AND METHOD

(57) Abstract: Disclosed are an apparatus and method for two-dimensional electrophoretic separation and subsequent isolation of analytes of interest, particularly polypeptides. The apparatus includes a sample separation cavity comprising a first electrophoresis region for performing charge and/or sized-based electrophoresis in a first dimension, and a second electrophoresis region for performing electrophoresis in a second dimension, in a direction substantially perpendicular to the first dimension. The second region also contains a plurality of elongate separation channels substantially perpendicular to the first dimension, and these channels contain a removable solid phase effective to immobilize separated components upon activation. Non-immobilized components can then be removed, and immobilized components recovered, without loss of resolution.

ELECTROPHORESIS APPARATUS AND METHOD

Field of the Invention

The present invention relates to methods and apparatus for electrophoretically separating and isolating components of a multicomponent sample. In particular, it relates to methods of isolating selected components without loss of resolution after two-dimensional electrophoresis.

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Background of the Invention

For decades, electrophoretic separation methods have been central to identifying and characterizing chemical and biochemical samples. For complex samples, multidimensional electrophoresis methods have been employed to better separate species that comigrate when only a single electrophoresis dimension is used. The conventional approach to two dimensional electrophoresis is to perform the first dimension in a rigid, usually crosslinked matrix. For analysis of proteins, for example, the sample is usually fractionated first by isoelectric focusing (IEF) in a tube or strip gel to exploit the unique dependence of each protein's net charge on pH. Next, the gel containing the separated proteins is extruded from the tube, dried (these two steps can be bypassed using a strip gel) and laid horizontally along one edge of a slab gel, typically a crosslinked polyacrylamide gel containing sodium dodecylsulfate (SDS). Electrophoresis is then performed in the second dimension, perpendicular to the first, and the proteins separate on the basis of molecular weight.

A significant drawback of these traditional methods for two-dimensional electrophoresis is that two separate devices are needed to accomplish electrophoresis in the two dimensions. Moreover, traditional methods are susceptible to significant run-to-run variation because of variability in standard IEF and SDS gels, which cannot be re-used.

An improved two-dimensional electrophoresis apparatus and method were disclosed by Wiktorowicz and Raysberg in U.S. Patent No. 6,013,165 and in PCT Publication No. WO 99/61901, both of which are incorporated by reference. The method employs a single separation apparatus for electrophoresis in both dimensions and a flowable (liquid-state) separation medium that can be easily replaced with fresh media, so that a single apparatus can be used repetitively for multiple samples. The lack of a rigid gel also permits recovery of selected molecules by allowing the flow of separation medium through the plate. For example, by attaching microvalves at the terminus of each channel, the contents of a given channel can be recovered independently of adjacent channels.

Within a given channel, however, effective discrimination between sample components, and reproducibility of results, lies in the maintenance of the high degree of

resolution generated during second-dimension electrophoresis. Mobilization of bands within a given channel by pressure , *i.e.* by initiation of flow, leads to a loss in resolution, due to the unavoidable parabolic flow profile. In order to maintain the high resolution created by second-dimension separation in the channels, recovery of selected components requires non-pressure-driven methods.

Summary of the Invention

In one aspect, the invention provides a two-dimensional electrophoresis system for separating and recovering components within a sample. The system includes:

(a) an electrophoresis plate assembly which defines a sample separation cavity, bounded by a lower plate and an opposing upper plate, the cavity comprising

(i) a first electrophoresis region containing a first flowable aqueous medium, and adapted to perform charge and/or size-based electrophoresis in a first dimension; and

(ii) a second electrophoresis region, abutting the first electrophoresis region, containing a second flowable aqueous medium in physical communication with the first medium, and adapted to perform electrophoresis in a second dimension, in a direction substantially perpendicular to the first dimension.

The second region also contains a plurality of elongate separation channels defined by the lower plate and substantially perpendicular to the first dimension, and the channels contain a removable solid phase effective to bind and immobilize separated components following the second-dimension electrophoresis.

Also included within the system are (b) electrode means for generating a first voltage potential across the first electrophoresis region, and (c) electrode means for generating a second voltage potential across the second electrophoresis region. In the system, the media are such that the rates of migration of sample components in the second dimension depend on sample properties that are different from the sample properties that determine sample migration rates in the first dimension. In one embodiment, the flowable medium occupying the first region includes a plurality of linear polyacrylamide molecules, and the second electrophoresis region contains an isoelectric focusing region, having a continuous pK_a gradient immobilized on at least one of the major opposing surfaces, and contains a flowable low ionic strength aqueous buffer.

In one embodiment, the solid phase within the channels of the second elecotrophoresis

region comprises a plurality of solid particles. These particles are preferably composed of an uncharged polymer, such as crosslinked polystyrene (styrene/divinylbenzene copolymer) or a poly(alkyl methacyrlate), such as polymethylmethacrylate.

The solid phase contains a binding reagent having an activatable group effective to bind the components to the solid phase upon activation, preferably upon exposure to radiation. Typically, the binding reagent contains a photolabile group, such as an azide, a diazo group, or a benzophenone, which becomes reactive with the separated components upon photolysis. Accordingly, in this embodiment, the system also includes means for exposing the solid phase to irradiation effective to photolyze the photolabile group. Preferably, the exposing means is effective to selectively irradiate sites within the second electrophoresis region, *i.e.* within the channels, containing selected separated components. The exposing means may be an optical device of sufficiently narrow diameter to minimize cross-irradiation, *e.g.*, a fiber optic cable, or a light source in combination with a mask which permits exposure of selected regions. Very high resolution of irradiation can be achieved, if necessary, by employing high resolution masking methods well known in the art, such as photolithographic methods.

The binding reagent attached to the solid phase preferably further comprises a linkage, between the activatable group and the solid phase, which is not labile under the conditions of activation, but is labile under other selected conditions. Preferably, the linkage is a chemically cleavable linkage, such as a disulfide linkage, an azo linkage, an ester, a glycol, or a sulfone.

In another aspect, the invention provides a method for separating and recovering components within a sample. The method includes the steps of:

(a) providing a planar substrate defining a planar sample separation cavity which includes:

(i) a first electrophoresis region, containing a first flowable aqueous medium, and adapted to perform charge and/or size-based electrophoresis in a first dimension, and

(ii) a second electrophoresis region, abutting the first electrophoresis region, containing a second flowable aqueous medium, in physical communication with the first medium, and an immobilized continuous pKa gradient, and adapted to perform isoelectric focusing in a second dimension, in a direction substantially perpendicular to the first dimension,

wherein the substrate further defines, within the second electrophoresis region, a

plurality of elongate separation channels substantially perpendicular to the first dimension, containing a removable solid phase effective to immobilize the components following the second-dimension electrophoresis;

(b) applying the sample mixture to the first region;

(c) applying a first voltage potential across the first region, under conditions effective to cause the sample components to migrate across the region, such that different components become separated at least partially on the basis of size;

(d) applying a second voltage potential across the second region, such that the migrated sample components migrate into the second region, in a direction substantially perpendicular to the first dimension, and become separated on the basis of their isoelectric points;

(e) activating selected regions of the solid phase such that sample components become immobilized thereto, in regions of the activating;

(f) removing non-immobilized components, typically by washing the solid phase; and

(g) recovering the immobilized components.

Preferably, the solid phase comprises a plurality of solid particles contained within the channels. The particles are derivatized with a binding reagent having an activatable group effective to bind the components to the solid phase, upon the activating of step (e) above. In one embodiment, the binding reagent contains a photolabile group, such as an azide, a diazo group, or a benzophenone, which becomes reactive with the components upon photolysis, and the activating comprises irradiating the solid phase with radiation of a wavelength effective to photolyze the group.

Preferably, the binding reagent also includes a labile linkage, between the activatable group and the solid phase, which is not labile under the conditions of activation (*e.g.* photolysis). Examples include a disulfide linkage, an azo linkage, an ester, a glycol, and a sulfone, all of which are chemically cleavable. The immobilized components can be recovered from the solid phase by cleaving this labile linkage.

The method can be used to separate a variety of types of sample components, including polypeptides, glycopolypeptides, proteoglycans, nucleic acids, charged polysaccharides, and synthetic polymers. In a preferred embodiment, the components are polypeptides.

These and other features and advantages of the invention will become more clear from the following detailed description together with the appended drawings.

Brief Description of the Drawings

Fig. 1 illustrates an exemplary plate assembly that can be used in accordance with the invention, including a liquid loading region, a plurality of separation channels, and an elongate sample transport channel;

Fig. 2 shows a perspective view of the assembly of Fig. 1;

Fig. 3 shows a cross-section of the channel region of the assembly from Figs. 1 and 2;

Fig. 4 illustrates the derivatization of a solid particle with a binding reagent, binding of a component protein via photolysis of a photolabile group, and subsequent cleavage from the solid particle;

Figs. 5A-5D show exemplary modifications of the triangular region of the device from Fig. 1;

Figs. 6 and 7 show overhead and perspective views of another exemplary plate apparatus that can be used in accordance with the invention;

Fig. 8 shows an enlarged view of sample loading channels of the apparatus from Figs. 6-7;

Fig. 9 illustrates a modification of the apparatus of Figs. 6-8; and

Fig. 10 illustrates an enlarged view of an electrode reservoir from Fig. 9.

Detailed Description of the Invention

The present invention is directed to methods and apparatus for conducting multidimensional electrophoretic separations of sample mixtures within a single separation cavity and subsequently isolating components of interest. Analysis is highly reproducible and quantitative. The methods are particularly suitable for differential analysis of protein mixtures, where identification of a relatively few components in a mixture of many proteins is desired.

The invention provides, in a multidimensional electrophoresis apparatus having isoelectric focusing channels within the second dimension, such as described in U.S. Patent No. 6,013,165 and PCT Pubn. No. WO 99/61901, means for selectively and reversibly immobilizing resolved components of interest within the focusing channels. Non-immobilized components can then be removed, and the immobilized components recovered, as described further below. The method allows components to be selectively

recovered from a 2D separation and eliminates the necessity for maintaining the resolution obtained via the 2D separation during subsequent analysis.

I. Apparatus

A. Plate Assembly

In one aspect, the invention provides an apparatus for conducting two-dimensional electrophoretic separation and isolation of selected analytes, particularly polypeptides. The apparatus, a modification of that described in U.S. Pat. No. 6,013,165 and PCT Pubn. No. WO 99/61901, includes a plate assembly that defines a cavity bounded by opposing major first and second surfaces, each having a defined width and length. These major surfaces are spaced apart by an interfacial distance substantially shorter than the width and length of the cavity. The cavity further comprises (1) a first electrophoresis region, located along the upper portion of the cavity, and containing a first flowable aqueous medium, for performing charge and/or size-based electrophoresis in a first dimension along this upper portion, and (2) below the first electrophoresis region, a second electrophoresis region, containing a second flowable aqueous medium, in fluid communication with the first medium, for performing electrophoresis in a second dimension, in a direction substantially perpendicular to the first dimension. The basis of migration of sample components in the second dimension depends on sample properties (*e.g.*, molecular weight, molecular shape, hydrophobicity and/or hydrophilicity, and/or charge) that are different from the sample properties that determine the basis of migration in the first dimension.

The 2D separation method and apparatus is adaptable to a variety of separation conditions, including conditions for (1) isoelectric focusing, and (2) denaturing or non-denaturing size-based separations in flowable sieving media. Moreover, since electrophoresis is accomplished in both dimensions with flowable media, the media can be replenished after each sample separation without having to separate the plates.

Figs. 1 and 2 show an overhead view and perspective view, respectively, of an electrophoresis plate assembly 110 that can be used in practicing the present invention. A pair of plates 120,122 are disposed such that inner plate surfaces 120a and 122a are juxtaposed face-to face to form an enclosed separation cavity 124, for holding separation media. Plate 120, which is referred to arbitrarily as the bottom plate, defines a recessed region 126 which defines five of the six walls of cavity 124, as well as a plurality of separation channels 170.

In the assembled apparatus (plate assembly), plates 120 and 122 are joined together by any suitable means sufficient to ensure a liquid-tight seal with respect to separation cavity 124. For example, glass plates can be fusion-welded together using methods known in the art, *i.e.*, by holding the opposing faces of the plates together at an elevated temperature that is below the softening point of the plates, such that the inner surfaces 120a and 122a of the plates become bonded together. Alternatively, the plates can be joined together by anodic bonding, or simply by using one or more clamps along the edges of the plates.

As seen with reference to Fig. 2, region 126 further includes a first sample separation surface, designated surface 126a, for electrophoresis along the lateral dimension of this region, and a second sample separation surface 126b, for electrophoresis in a direction perpendicular to the first-mentioned dimension. Plate 122, which is referred to arbitrarily as the cover plate, includes an inner surface 122a which provides the sixth wall of separation cavity 124. As described further below, the cover plate is preferably transparent, or can be made transparent, to selected wavelengths of visible and/or UV light. Preferably, the surfaces 126a and 126b of recessed region 126, and the inner surface 122a of cover plate 122, are substantially planar, to facilitate the creation of undistorted electric field lines and enhance sample separation during electrophoresis.

In the embodiment shown in Figs. 1-2, region 126 also encompasses a triangular liquid loading region 160 at the upper end of the plate, for conveniently introducing and removing separation media to and from the separation cavity before and after electrophoresis. Region 160 is also useful for forming a pKa gradient coating on the inner surfaces of the plates for isoelectric focusing, and/or for loading a slurry of solid particles into the second separation region, as described below.

As seen in Fig. 1, and particularly with reference to Fig. 2, plate 122 defines, in its lower left-hand corner, a sample loading port 130, at or through which sample is introduced into the separation cavity, and which also provides access to the separation cavity for a first electrode 130a (not shown) for establishing a voltage potential at that site. Electrodes can be made of any appropriate conductive material, such as platinum, nichrome, or gold, etc., with platinum being preferred. An electrode port 132 is defined in the upper right-hand corner of plate 122, for providing a second electrode 132a (not shown) in electrical contact with the separation cavity. The first and second electrodes are used to perform electrophoresis of a sample along a first dimension extending from port 130 to port 132, to

generate a series of separated sample components along the upper edge of 126a after the first electrophoresis step is complete. Port 135 in plate 122 is included for transporting separation media and wash fluids into and out of the separation cavity, and also for forming the IEF coating gradient.

To facilitate sample loading, port 130 can be accompanied by a waste port 131 defined in plate 122, such that both ports are in fluid communication with elongate sample transport channel 180. This allows a precise amount of sample to be injected into channel 180, as discussed further below.

Plate 120 also contains an elongate sample transport channel 180 extending from the lower left hand area of the plate to the upper left hand corner of surface 126a, for size-based electrophoretic separation of the sample during transit to separation cavity 124. Channel 180 preferably has a depth in plate 120 equal to the depth of recess 126. The width of channel 180 is preferably no more than about 10 times the channel's depth, and is preferably equal to or less than about 5 times the channel depth.

Plate 122 also contains an elongate slot 140 which extends laterally along the lower edge of the plate, to provide a passageway for ingress and egress of separation media and wash solutions into and out of the separation cavity before or after electrophoresis.

Plates 120 and 122 further define channel egress ports 136 and 138, respectively. These ports can be used to collect resolved sample components individually from one or more channels after the second electrophoresis step and immobilization of selected components, as described below. In operation, the ports remain closed until immobilization is complete, to minimize turbulence and maintain resolution of components. In the embodiment illustrated in the Figure, each plate contains an egress port for alternating channels, so that ports 136 and 138 are staggered relative to each other. The staggering of egress ports, while not required, facilitates the connection of capillary tubes to the ports for collecting fluids from each channel.

With continued reference to Figs. 1 and 2, surface 126b comprises a plurality of parallel separation channels 170 aligned in a direction perpendicular to the bottom edge of the plate. In a preferred embodiment, in which second dimension separation is based on isoelectric focusing (IEF), surface 126b is further characterized by the presence of a plurality of buffering moieties (*e.g.*, the "Immobiline ®" compounds sold by Amersham-Pharmacia Biotech, Uppsala, Sweden), which define a pKa gradient leading away from

region 126a. Each channel 170 is separated by a partition 172 (Fig. 3), preferably having a height flush with inner surface 120a of plate 120, in order to form liquid-tight seals between the channels when plates 120 and 122 are assembled together. The depths of the channels are preferably the same, and are also preferably the same as the depths of regions/surfaces 126a and 160.

The number of channels 170, and their dimensions, will vary depending on sample complexity and the desired resolution. Generally, sample resolution will increase as the number of channels is increased, subject to the limit of resolution achieved by electrophoresis in the first, lateral, dimension. Preferably, the channels are dimensioned so that the channel resolution is at least twice the sample resolution in the lateral dimension of the separation medium, so that each sample band partitions into from one to three channels.

B. Removable Solid Phase

In accordance with the invention, each channel 170 contains a removable solid phase which is effective to bind and immobilize any selected component, in response to an external activation, following second-dimension electrophoresis. The solid phase preferably consists of a plurality of solid particles or beads, such as illustrated at 171 in Fig. 3, each preferably about 5-10 μM in diameter, typically forming a layer having a depth of about 2 to 5 beads in each channel. (Note that Fig. 3 is not drawn to scale; preferred dimensions for the channels are described in Section D below.) Such beads are easily installed and then removed from the channels, as described below. However, it is also possible that a continuous solid phase could be used, *e.g.* a strip within each channel; or surface 126b may comprise a funtionalized removable surface, such as a membrane, which is segmented via impermeable barriers into channels.

The solid phase is composed of any material that does not interfere with electrophoretic separation and can be chemically functionalized. Preferred materials are uncharged polymers, such as crosslinked polystyrene (styrene/divinyl benzene copolymer) or a poly(alkylmethacrylate), such as polymethylmethacrylate.

For use with beads, a dam may be provided at the outlet of the channels to serve as a packing surface and prevent the beads from flowing out of the channels. However, it has been found that beads of polymeric materials such as noted above settle stably within the channels, so that a dam is generally not necessary.

The solid phase is derivatized with a binding reagent having an activatable group

effective to bind selected separated components upon activation. The activation is most conveniently exposure to radiation of an appropriate wavelength range. Thermally or chemically activatable groups could be used in applications where localization of activation is less critical.

Preferably, the binding reagent contains a photolabile group which is converted, upon photolysis, to a group which is reactive with the sample components. For example, photolysis of an azide generates a nitrene, a broadly reactive species which reacts with a variety of groups, including insertion into C-H and other bonds. Other photolabile groups include diazo compounds, which generate carbenes, another highly reactive species. However, these tend to be more reactive with ambient water than nitrenes (see Ji & Ji, 1989, for a review of photoreactive heterobifunctional reagents).

Also well known are photoreactive protecting groups for various functional groups such as alcohols, amines, and carboxylic acids. For example, ortho-nitrophenyl ethers are used as alcohol protecting groups. However, the species regenerated on photolysis of these groups (*e.g.* an alcohol) are typically not as reactive as the species described above.

The system also includes, in one embodiment, means for selectively exposing selected regions of the channels to radiation effective to cleave a photolabile linkage. A region containing a single component can be irradiated individually via an optical device of sufficiently narrow diameter to minimize cross-irradiation, *e.g.*, a fiber optic cable. Very high resolution of irradiation can be achieved, if necessary, by employing masking methods such as those described for use in combinatorial synthesis (Fodor *et al.* (1991); U.S. Patent No. 5,143,854 (1992)).

Preferably, the binding reagent also contains a labile linkage, between the activatable group and the solid phase, which is not labile under the conditions of activation of the first group. See, for example, the binding reagent illustrated in Fig. 4, which contains a disulfide linkage. Accordingly, once the regions of the solid phase containing immobilized components have been separated from non-immobilized components, this second linkage may be cleaved to release the selected components from the solid phase. In a preferred embodiment, this labile linkage is chemically cleavable. Examples include disulfides (cleavable by reduction, typically using dithiothreitol), azo groups (cleavable with diothionate), sulfones (cleavable with basic phosphate, with or without dithiothreitol), glycols, cleavable by periodate, and esters, cleavable by hydrolysis. Alternatively, the

second labile linkage may be a photolabile linkage cleaved at a different wavelength from that used to photolyze the first group.

The binding reagent may include any combination of a photoreactive and non-photoreactive (*e.g.* chemically) cleavable component. For example, Oatis *et al.* (1998) describe a reagent in which the photoreactive portion is a benzophenone and the chemically cleavable linkage is an ester; Jaffe *et al.* (1980) describe reagents having a azo functionality (photocleavable) and a diazo linkage (chemically cleavable). Use of other binding reagents containing linkages cleavable under different conditions are within the knowledge of those skilled in the art.

An exemplary reagent containing a photolabile (azo) linkage and a disulfide linkage is N-succinimidyl-3-[4-azidophenyl]-dithio propionate (SADP). This and related compounds are described in Vanin and Ji, 1981.

Figure 4 illustrates derivatization of a solid substrate with this reagent. The solid substrate, such as beads of crosslinked polystyrene or PMMA, is treated according to known methods to provide reactive surface groups. Derivatized resins are also commercially available, *e.g.* aminomethylated polystyrene/divinylbenzene and (amino-terminal) polyethylene glycol-derivatized polystyrene/divinylbenzene. The N-succinimidyl ester of SADP is highly reactive to attack by nucleophiles; accordingly, it reacts with amine surface groups to generate an amide linkage, with displacement of N-hydroxysuccinimide. Any excess amine surface groups may be capped, *e.g.* by acylation, to eliminate potentially charged groups on the polymer surface. The polymer is thus derivatized with a binding reagent containing a photolabile group (the terminal aryl azide) and a chemically cleavable linker (the disulfide) intervening between this group and the solid support.

C. Alternative Configurations

Figs. 5A-9 illustrate additional embodiments that may be utilized in the devices of the present invention, particularly for maintaining a relatively uniform electrical field during electrophoresis in the first dimension. Fig. 5A shows a modification in which triangular liquid loading region 160 from Fig. 1 can be modified to include a vertical barrier 150 that extends from beneath port 135 to the top of surface 126a. A benefit of barrier 150 is that during the first dimension of electrophoresis, the electric field lines are constrained to the region bounded by surface 26a, so that band distortion may be reduced.

Fig. 5B shows a modification in which one of plates 120 and 122 contains an

additional port 152 which is closable with a suitable plug (not shown). After regions 126a and 126b have been filled with a desired medium or media, a low conductance medium that preferably has an ionic strength at least 5 times, and more preferably at least 10 times lower than that of the surrounding medium, is introduced into triangular region 160 through port 152 with egress through port 135, so that a vertical "wall" or region of low conductance medium is created in region 160 between ports 152 and 135. After loading of the low conductance buffer, ports 135 and 152 are closed, and electrophoresis is performed as described herein. Field lines in the first dimension of electrophoresis are thus constrained to region 126a.

In Fig. 5C, the first electrophoresis region includes a trench 154 extending across the lateral dimension of region 126 and which provides a deeper cross-section for this region relative to region 126b (*e.g.*, twice as deep as the region 126b). After regions 126a and 126b have been filled with a desired medium or media, region 126a (trench 154) is preferably filled with a high conductance/high viscosity medium (*e.g.*, due to the presence of a selected polymer) via port 130 or 131 and port 132, to help constrain electrical field lines to region 126a during electrophoresis in the first dimension. By providing a greater cross-section than the cross-sections of adjacent regions 160 and 126b, trench 154 also helps limit mixing between regions 126a and 126b.

Fig. 5D shows another modification in which plate 122 additionally includes a horizontal slot 156 located just above region 126a. Initially, slot 156 is filled with a plug (not shown) which closes the slot and has an inner surface that is flush with the inner surface of plate 122. After the inner surfaces of regions 126a and 126b have been prepared as described herein, and after the chamber has been filled with the desired electrophoresis medium or media, the plug is pressed further through the slot until the end of the plug snuggly contacts surface 126a of plate 120, thereby creating a horizontal barrier across the top of region 126a which constrains electric field lines during the first dimension of electrophoresis.

Figs. 6-12 illustrate another, generally preferred, plate configuration in which the triangular region from Fig. 1 is moved to the other end of the device, adjacent the edge of the second electrophoresis region. Apparatus 200 includes a bottom plate 220 and top plate 222. Bottom plate 220 includes a first sample separation surface 226a, defining a first electrophoresis region 226a, for electrophoresis along the lateral dimension of this region,

and a second sample separation surface 226b, for electrophoresis in a direction perpendicular to the first dimension. Plate 220 further defines a lateral channel 232a for providing fluid communication with port 232 in plate 222.

Plate 220 also contains an elongate sample transport channel 280 extending along the left edge of region 226b. Channel 280 terminates with a peripheral channel 231a which links port 230a to channel 280. Peripheral channels 231b, 231c, and 231d meet at junction 231e (see Fig. 8), and are linked in fluid communication with channel 280 via channel 231f. Channels 231b, 231c, and 231d place ports 230b, 230c, and 230d in fluid communication with channel 280. The operation of these channels in introducing sample is discussed further below. Also, it will be appreciated that any other suitable channel arrangement can be used in the apparatus to facilitate loading of sample.

As above, surface 226b comprises a plurality of parallel separation channels 270 aligned in a direction perpendicular to the first direction of electrophoresis, separated by partitions 272, and containing a removable solid phase as described above. At the end of those channels, plate 220 further defines a triangular liquid loading region defined by surface 260, for conveniently introducing and removing liquids into and from the separation cavity. Triangular region 260 is bordered at its lateral edges by channels 233a and 233b, which provide fluid communication with ports 234a and 234b, respectively, in plate 222. Port 235 in plate 222 provides a convenient site for transporting separation media and wash fluids into and out of the separation cavity. It can also be used for forming an IEF coating gradient, and subsequently for loading a slurry of derivatized beads, in region 226b, as detailed below.

Plates 220 and 222 further define pluralities of alternating channel egress ports 236 and 238, for collecting resolved sample components individually from one or more of channels 270, as with apparatus 100 above. As above, in operation, the ports remain closed until immobilization of selected sample components is complete, to minimize turbulence and maintain resolution of components.

The other various ports, particularly ports 230a, 230b, 230c, 230d, 232, 234a, and 234b, may also be provided with electrodes in order to control movement of sample (ports 230a-d), for electrophoresis in the first dimension (ports 230a and 232), and for electrophoresis in the second dimension (ports 230a, 232, 234a and 234b).

D. Dimensions

For use in a typical biomolecule separation, *e.g.* differential analysis of a protein sample, the separation cavity preferably has a length dimension of about 1 to 20 cm (*e.g.*, 12 cm); a width dimension of about 1 to 50 cm (*e.g.*, 10 cm); and a depth dimension (interfacial distance between the major opposing surfaces of plates) of about 50 to 200 μm (*e.g.*, 100 μm); the first electrophoresis region preferably has a path width of about 0.1 to 2 cm (*e.g.*, 0.25 cm); channels 170 preferably have widths of about 0.25 to 1 mm (*e.g.*, 0.67 mm) each and depths that are preferably the same as the above-mentioned interfacial distance, and are spaced apart by partitions having a width of about 0.1 to 0.5 mm (*e.g.*, 0.33 mm); elongate channel 180 preferably has a length of about 0.5 to 15 cm (*e.g.*, 12 cm), a width of about 0.2 to 1 mm (*e.g.*, 0.5 mm), and a depth that is preferably the same as the above mentioned interfacial distance); slot 140 preferably has a length spanning all channels, and a width of about 0.5 to 3 mm (*e.g.*, 2 cm); ports 130, 131, 132, 135 and 136,138 preferably have diameters of 0.5 to 3 mm (*e.g.*, 1 mm); and ports 130 and 131 are spaced apart by a center-to-center distance of 0.5 to 2.5 cm (*e.g.*, 1.2 cm). Of course, dimensions outside the above preferred dimensions can also be used.

E. Materials

Plates 120,122 (or 220, 222) can be formed of any material suitable for electrophoresis of the selected sample. Preferably, at least one of the plates is formed of a material that is transparent to wavelengths of irradiation used to activate the activable group of the binding reagent and to visualize or locate sample components in the separation cavity. The plates can be conveniently formed out of a silicon dioxide-based glass, such as borosilicate, although plastics, *e.g.* polycarbonate, or quartz are also contemplated. Quartz, while less amenable to microfabrication than glass, is favored for photocleavage of certain binding reagents which is most effective at lower wavelengths (<300 nm).

The inner surfaces of the separation cavity are preferably inert with respect to the sample, to minimize adsorption of the sample to the inner surfaces during electrophoresis. Such adsorption is generally undesirable because it can disrupt band resolution particularly in the first dimension of electrophoresis.

Additionally, materials such as silicate glasses tend to have charged groups on their surfaces that can cause electroendosmotic flow (EOF) of the separation medium during electrophoresis. EOF is a phenomenon in which a bulk flow of the electrophoresis medium

arises due to the effect of the electric field on counterions adjacent to charged surfaces of a separation cavity. In the case of a surface that is negatively charged, such as a silicate glass surface, there is a build-up of positive counterions (cations) in the solution adjacent to the surface. In an electric field, this shell of cations can cause the medium to migrate toward the cathodic electrode at an EOF rate dependent on the thickness of the cationic shell.

The rate of EOF can provide an important variable that can be optimized to improve the separation of two or more closely migrating species. In particular, when electrophoresis is carried out under conditions in which EOF and the migration of species to be separated are in opposite directions, the effective path length for separation can be made extremely long by making the rate of EOF in one direction nearly equal to the electrophoretic migration rate of the analyte attracted most strongly in the opposite direction by the electric field. In the present invention, EOF may or may not be desirable for the first dimension of electrophoresis, depending on the nature of the sample and the degree of desired separation. However, for the second (IEF) dimension of electrophoresis, EOF is preferably avoided so that the uniformity of the IEF pH gradient is not disturbed.

If the materials from which the plates are made are not inherently sufficiently inert towards the sample, the inner surfaces of the plates and all other inner surfaces of the separation cavity can be coated with any suitable coating material, to reduce sample adsorption to an acceptable level. Since electrophoresis is usually performed in an aqueous separation medium, adsorption of sample can usually be reduced by covering the inner surfaces of the separation cavity with a hydrophilic coating that masks potentially adsorptive surface regions.

Exemplary reagents for coating adsorptive surfaces include polyacrylamide, polyvinyl alcohol, polyethers, cellulose acetate, polyalkylene oxides, poly(vinylpyrrolidone), and other materials as are known in the art. Preferably, such coatings are attached to interior surfaces covalently, although coating by adsorption may also be suitable.

Coating reagents for reducing sample adsorption can also be used to control the magnitude of EOF. For example, EOF along glass silicate surfaces can be substantially reduced by coating them with a neutral reagent that masks a substantial percentage of surface silanol groups. The magnitude of EOF can be further controlled by using coating reagents that include positively or negatively charged groups. Positively charged coatings can be used to nullify surface negative charges to give a net surface charge of zero, so that

EOF = 0. Coatings with higher positive charge densities can be used to reverse the direction of EOF for charged surface materials. This can be useful for slowing the net migration rates of positively charged sample species. Conversely, negatively charged coatings can be used to impart to or increase the magnitude of negative charge on surfaces, to slow the net migration rates of negatively charged species. Representative positively charged coatings include polyethyleneimine, quaternized polyethyleneimine, and chitosans, for example. Representative negatively charged coatings include carboxylate and sulfonate containing materials, such as poly(methylglutamate) and 2-acrylamido-2-methylpropanesulfonate polymers, for example. It will be recognized that charged coatings can also effectively reduce sample adsorption, especially for samples having the same charge polarity as the coating.

F. Preparation of Immobilized pKa Gradient

According to a preferred embodiment, the second electrophoresis region includes an isoelectric focusing region that contains a pKa gradient immobilized on at least one of the major opposing surfaces, for isoelectric focusing in a direction substantially perpendicular to the first dimension. The pKa gradient is effective to produce an isoelectric focusing pH gradient when the apparatus is filled with an aqueous medium, to promote migration of sample components to locations in the gradient where the local pH is equal to the pI of each component.

The immobilized pKa gradient is formed on one or both major inner surfaces by any method suitable for forming a gradient having a desired pKa range, resolution, and buffering capacity. Suitable methods for forming such gradients are described in co-owned U.S. Patent No. 6,013,135, which is hereby incorporated by reference in its entirety. Generally, forming the pKa gradient entails exposing plates to a solution containing a gradient of buffering molecules, under conditions effective to promote covalent attachment of molecules to the plates, such that the pKa gradient of the solution is transferred to the plates. In one approach, the immobilized pKa gradient is formed by pumping a solution containing a gradient of such molecules into the bottom of a vertically oriented separation cavity.

The buffering groups for creating the IEF gradient are attached to the plates by any suitable method known in the art. A large number of buffering compounds have been developed for creating IEF gradients. In particular, a variety of buffering compounds

having reactive groups suitable for covalent attachment to solid phase surfaces are commercially available (*e.g.*, the "Immobiline®" compounds sold by Amersham-Pharmacia Biotech, Uppsala, Sweden). For silicate glass plates, buffering compounds can be attached directly to surface silanol groups, as reviewed in Li (1992), or they can be attached via an intermediary coating that provides other reactive groups.

The sample capacity of the isoelectric focusing region will depend in part on the buffering capacity of the immobilized buffering groups on the major inner surface(s) of the plates. Buffering capacity generally increases as the density of buffering groups on the surface is increased. Thus, it is preferable to attach as high a density of buffer groups to the surface as possible, to allow the plates to accommodate higher concentrations of sample components to be separated on the basis of pI.

The plate surfaces above and/or below IEF region 126b can be coated with buffering molecules defining a selected pKa, to stabilize the pKa gradient in the IEF region. Preferably, the pKa for the coating is selected to be outside the pKa range defined by the IEF gradient. U.S. Patent No. 6,013,165, cited above, provides a protocol for coating the triangular region and sample transport channel of apparatus 100 (Fig. 1) with Immobilines® having an average pKa of about 3.5. The presence of this coating can help stabilize the anodic end of the IEF gradient. A similar procedure can be used to coat the plate surfaces at the cathodic end of the IEF gradient, if desired, using suitably basic Immobilines®.

II. Separation and Isolation Method

The invention provides a method for separating and isolating one or more components of a sample mixture, using an electrophoresis apparatus such as described above. The method is useful for identifying and characterizing a variety of samples, and particularly for differential sample analysis, as in diagnostic detection of particular molecules in a biological sample, or for monitoring changes in sample composition over time.

A. Preparation of Sample

The sample can be any substance for which electrophoretic separation may be useful. Preferred analyte types include polypeptides, glycopolypeptides, proteoglycans, nucleic acids, charged polysaccharides, and synthetic polymers, although other substances, especially from biological sources, are also contemplated. The sample may be derived from cellular or tissue extracts (*e.g.*, Anderson *et al.*, 1991), or biological fluids, such as blood,

urine, semen, synovial fluid, saliva, or fractions thereof, prepared by known methods.

If necessary, the sample components can be modified to include one or more detectable labels to facilitate detection and quantification in the separation medium. Such labeling is preferably done prior to separation, to minimize disruption of resolution after separation. In one approach, the sample is labeled with a fluorescent label, such as a fluorescein, rhodamine, eosin, or "BODIPY™" group, according to methods well known in the art. The reactive functionality on the label is selected to ensure labeling of most or all of the components of interest in the sample. Preferably, the label contains a reactive functionality that reacts with a limited set of complementary reactive groups. For proteins, for example, cysteine-selective reagents, such as iodoacetamide and maleimide functionalities, are preferred, since most proteins (≈ 90-95%) contain at least one cysteine residue. Tyrosine (*i.e.* phenol) and amine-reactive labels can also be used. Generally, hydrophilic labels are preferred, to help avoid sample precipitation. Fluorescent compounds suitable for labeling proteins and the like are well known, and are commercially available from Sigma Chemical Co. (St. Louis, MO) and Molecular Probes, Inc. (Eugene, OR). Preferred derivatized labels include functionalized eosin, "BODIPY"™, and monobromobimane. A chemical labeling reaction is carried out for a time sufficient to label uniformly most or all labelable components in the sample. Unbound label can be removed by quenching with an excess amount of a scavenger substrate, such as free cysteine, followed by passing the reaction mixture through a size-exclusion gel, such as Sephadex™ G-25 or G-50 (Amersham-Pharmacia Biotech).

Although fluorescent derivatization of sample components may alter the pI values of some components, such alterations are acceptable since they do not interfere with detecting and monitoring of the components.

Alternatively, the sample may include a detectable radioisotope, such as ^{125}I, ^{32}P, ^{35}S, ^{14}C, or ^{3}H. Chemical and biochemical methods for introducing such isotopes into samples are well known in the art.

B. Selection and Loading of Separation Media

In operation, a plate assembly is provided having the desired dimensions and containing a pKa gradient spanning a desired pI range in the isoelectric focusing region. For example, for analysis of proteins in a pI range of 4 to 9, the isoelectric focusing region contains a continuous buffer gradient spanning a pKa range of less than or equal to 4 to

greater than or equal to 9. The assembly is encased in a device that includes valved inlet/outlet ports and electrodes which form liquid-tight connections with corresponding ports and slot(s) in the cover plate.

Prior to sample loading and separation, the separation cavity of the system is filled with one or more flowable separation media. For embodiments that utilize IEF in the second dimension, the medium in the second electrophoresis region preferably has a low ionic strength, in order not to interfere with the IEF step, although soluble ampholines can also be included if desired to strength the buffering capacity of the pI gradient.

For separations in which the first dimension of electrophoresis is performed at an acidic pH, the pH of the medium is typically more acidic than the lowest pKa of the IEF region. This ensures that sample components that have pI values within the pH range of the IEF gradient will migrate into that region during the IEF step. Exemplary acidic buffers for the first dimension of electrophoresis include citrate, formate, and acetate, typically at a concentration of about 1 to 50 mM, and preferably about 5 to 20 mM.

To reduce precipitation of sample components during electrophoresis, and particularly during isoelectric focusing, the separation medium may additionally include one or more neutrally charged denaturing agents or detergents to reduce non-covalent interactions between sample molecules and wall interactions. Exemplary denaturing agents include urea, thiourea, and dimethylformamide (DMF). Exemplary neutral detergents include polyoxyethylene ethers provided under the trade name "Triton®", such as nonaethylene glycol octylcyclohexyl ether ("TRITON®" X-100), polyglycol ethers, particularly polyalkylene alkyl phenyl ethers, such as nonaethylene glycol octylphenyl ether ("NONIDET" P-40 or NP-40), polyoxyethylene sorbitan esters, such as polyoxyethylene sorbitan monolaurate ("TWEEN®"-20), polyoxyethylene esters, polyoxyethylene ethers, such as polyoxyethylene (23) lauryl ether ("BRIJ®"-35), N,N-bis[3-gluconamidopropyl]cholamide, decanoyl-N-methylglucamide, glucosides such as octylglucoside, and the like. Neutral zwitterionic detergents can also be used. The optimal concentration of a denaturing agent or detergent will depend on the particular detergent used. Urea is typically used at a concentration up to about 10M, for example, with a concentration of 4M to 8M being preferred. Generally, the detergent concentration will range from 0.01% to 5% (v:v), and more typically between 0.025 and 2%, although these ranges are not limiting.

Sample adsorption and EOF can also be adjusted by including suitable reagents in the separation medium and running buffers. For example, negative surface charges can be masked by including a cationic additive in the medium, such as metal amine complexes, amines and polyamines such as propylamine, triethylamine, tripropylamine, triethanolamine, putrescine, spermine, 1,3-diaminopropane, morpholine, and the like. Zwitterionic species comprising both negatively and positively charged groups that are isoelectric at the pH of electrophoresis can also be used, such as trialkylammonium propyl sulfonates, where alkyl is methyl, ethyl, propyl, etc. (Peterson *et al.*, 1992, Zhu *et al.*, 1990, Bushey *et al.*, 1989, and Chen *et al.*, 1992).

The separation medium may also include soluble agents for coating the walls of the separation cavity, to help reduce endosmotic flow (EOF) during electrophoresis. Such soluble coating agents include quaternary ammonium-containing polymers (Wiktorowicz (1990, 1991), methyl cellulose derivatives (Molteni *et al.*, 1994), cellulose acetate (Busch *et al.*, 1995), polyethylene oxide (Fung *et al.*, 1995), chitosan (Sun *et al.*, 1994), polyvinyl alcohol (Gilges *et al.*, 1994), polyethylene glycol (Wang *et al.*, 1992), polyethylenimine (Ibid.), and polyethylene oxide-polypropylene oxide-polyethylene oxide triblock copolymers (Ng *et al.*, 1994), for example. Typically, soluble coating agents can be included at concentrations of about 0.05% to about 4%, and more preferably of about 1% to about 2%.

The choice of additives in the separation medium will depend in part on the sample and the nature of the interior surfaces, as well as other factors. In some applications, it may be desirable to use both a covalent surface coating and soluble buffer agents to control sample adsorption and EOF.

The separation medium may also contain a polymeric material (also referred to as "entangled polymer") that differentially impedes sample components on the basis of their sizes. A variety of polymeric materials that promote size-based separation of analytes are known in the art, such as linear polyacrylamide (Werner *et al.*, 1993), polyethylene oxide (Schans *et al.*, 1994), dextran (Lauch *et al.*, 1993), polyethylene glycol (Ganzler *et al.*, 1992), and polyvinyl alcohol (Alfonso *et al.*, 1995). The appropriate concentration and size of the polymeric material included in the medium will generally depend at least in part on the physical properties and complexity of the sample being analyzed, the properties of the selected polymer(s), and the desired range of component molecular weights to be resolved.

For example, if only components with a high molecular weight are of interest, a higher concentration of polymer is used, which allows low molecular weight components to pass through quickly while larger components migrate more slowly. Preferably, the separation medium remains flowable, that is, substantially in liquid form, so that the medium can be easily removed from or replaced in the apparatus by moderate pressure differentials (*e.g.*, less than 50 psi). Further guidance regarding the choice of polymeric material, size and concentration can be found in the references cited above. In a preferred embodiment, the polymeric material is linear polyacrylamide, *e.g.*, 3% w/v with an average molecular weight (MW) of 100-300 kDa.

The inclusion of such polymeric materials is useful for enhancing the level of sample separation in the first dimension of electrophoresis, wherein sample components can be separated on the basis of a combination of their sizes and net charges. Such polymeric materials may also be useful for reducing convection currents and EOF in the separation medium during and after electrophoresis. A further advantage is that these polymers do not interfere with the isoelectric focusing step.

C. Loading of Removable Solid Phase

The removable solid phase within the channeled region, which is preferably a plurality of functionalized polymeric beads, as described above, can be conveniently installed by filling the channels within the second electrophoresis region with a slurry of such beads in a low ionic strength medium as described above, and allowing the slurry to settle. With reference to Fig. 6, a slurry of derivatized polymeric beads, as described above, in a low-ionic strength solution, preferably containing one or more denaturing reagents such as urea and thiourea, is loaded into the separation cavity via port 235, with egress through port 232, until residual air bubbles have been removed from the cavity, and the slurry is allowed to settle, typically for about 5 to 15 minutes.

The slurry typically contains about 10 weight percent beads. For an apparatus having dimensions as described in Section IF above, and using beads having a diameter of about 5-10 μ, this treatment typically deposits a layer a few beads in depth on the lower surface of the channels. This amount is found to be suitable for capturing selected components from the separation of a protein sample containing several thousand proteins. Sensitivity is high, allowing detection of picomole quantities or less; accordingly, a typical sample may contain about 10 nmoles of protein. A higher or lower quantity of beads may be used depending on

the quantity of protein or other analytes in the sample.

After settling of the slurry, port 235 is closed, and valves at ports 230 (any of a-d) and 232 are opened to admit an electrophoresis medium suitable for the first-dimension separation into region 226a. This medium is preferably a low pH, flowable entangled polymer solution as above, which preferably includes a denaturant, for effecting size-based separation in the first dimension. This loading is generally effective to displace any residual slurry that may be present in region 226a. However, the presence of a minor amount of residual beads is not expected to affect separation in region 226a. Some diffusion between the two solutions in the cavity may also occur at this stage, but again this should not significantly affect performance.

D. Sample Injection

The device preferably includes an elongate sample transport channel, such as channel 180 in Figs. 1 and 2 or channel 280 in Figs. 6-12, to lengthen the migration distance in the first dimension, and to increase spacing between bands.

With the plate equilibrated with the appropriate solutions, sample injection can be accomplished by hydrodynamic or electrophoretic means. Referring to the embodiment in Figs. 1 and 2 for illustrative purposes, hydrodynamic injection is performed by closing all ports and slot(s) except ports 130 and 131, and pumping a selected volume of sample through the injection chamber (*i.e.*, the portion of channel 180 located between ports 130 and 131). The sample can be moved into the portion of channel 180 beyond port 131 by closing port 131, opening port 132, and pumping the appropriate volume of buffer solution through port 130. The injection chamber can then be purged of residual sample by closing port 132 and opening port 131 again, to wash a selected amount of solution through the injection chamber. Electrophoretic sample injection can be accomplished by filling the injection chamber with a selected amount of sample via ports 130 and 131 as above, closing those ports, and then applying an electric field between ports 130 and 132 for a selected time (*e.g.*, 5 kV for 1 to 5 seconds) so that a small aliquot of positively charged sample (components with pI values greater than the pH of the medium) migrates into the separation channel upstream of port 131, and then optionally removing any residual sample via ports 130 and 131. Note that if desired, sample migration can be monitored, for example, by fluorescence detection.

The advantages of hydrodynamic over electrophoretic injection schemes are well-

known and are mainly concerned with the oversampling of faster migrating components in electrophoretic injection. Hydrodynamic injection does not suffer from this shortcoming since all components are injected within the solution. Higher sensitivity may be experienced with electrophoretic injection, however, since only sample molecules (not buffer or water) enter the separation path, and sample components are more highly concentrated at the start. However, sensitivity can be improved for hydrodynamic injection by using a sample buffer that has lower ionic strength than the buffer solution in channel 180, or by interposing a volume of low ionic strength buffer (preferably having an ionic strength at least 5 times, and more preferably at least 10 times lower than that of the surrounding medium) between the sample and the separation buffer, to promote sample stacking. The amount of sample injected for analysis will vary according to the complexity of the sample, the type of detection, etc. By way of illustration, a sample volume may consist of 40 nL of a 100 μg/mL sample mixture of 1,000-10,000 fluorescently labeled polypeptides.

With reference to the embodiment in Figs. 6-8, the configuration of ports 230a-230d and channels 231a-231d and 231f (Fig. 8) is useful for loading samples by different methods. In one approach (T-injector mode), sample solution is pumped into port 230b, with egress out of port 230d, so that sample is placed at junction 231e. The sample at junction 231e can then be moved into channel 180 by imposing an electric field between ports 230c and 232. After the sample has reached channel 180, the electric field between ports 230c and 232 can be replaced with a field between ports 230a and 232, to reduce leakage into channel 180 from channels 231b-231d. Preferably, channel 231f is preloaded with a low conductance buffer (conductance lower than that of the surrounding buffer) to promote sample stacking immediately downfield of the low conductance buffer when the sample reaches channel 180. In a second approach, sample is pumped into port 230b, with egress out of port 230c, in order to fill channel 231c with a selected amount of sample. Channel 231b is optionally purged of residual sample by pumping buffer into port 230d, with egress out of port 230b. An electric field is then applied between port 230c and port 232 to transport the sample into channel 180. Again, channel 231f is preferably preloaded with a low conductance buffer to promote sample stacking immediately downfield of the low conductance buffer when the sample reaches channel 180.

E. Separation Process

After sample loading is complete, electrophoresis is performed across region 226a (first dimension) by applying an electric field between ports 230a and 232 (*e.g.*, 5 to 30 kV), so that injected sample components migrate through channel 280 and into region 226a towards a cathodic electrode at port 232. The plate assembly is preferably maintained in a horizontal orientation, to minimize convection currents in separation medium. To control temperature, the plates can be placed on a constant-temperature heating/cooling device, such as a Peltier device, to maintain the separation medium at a selected temperature (*e.g.*, 0 to 40°C) and prevent overheating. The separation process may be monitored in real-time, *e.g.*, by fluorescence or chemiluminescence detection. A constant field or pulsed field can be used, depending on the sample and the desired resolution. The maximum field permissible is dependent on the ability of the device to dissipate Joule heat, which is typically facilitated by contact-cooling (*e.g.*, using a Peltier device) or by convective cooling (*e.g.*, high Reynolds number air-flow).

The first dimension of electrophoresis is usually completed within a few minutes, depending on the magnitude of the field. For example, cationic polypeptides can migrate approximately 20 cm within 10 minutes in a field of 250 V/cm. Longer electrophoresis times in the first dimension, or lower concentrations of entangled polymers, can be used to select for slower-migrating components.

When the fastest migrating component reaches port 232, or the desired amount of separation has occurred, the field is turned off, and a new field is applied across region 226 in a direction substantially perpendicular to the first dimension. This field can be generated by balancing the electric potentials at ports 230a and 232 to establish a substantially uniform field vertically across regions 226a and 226b towards region 260. In another approach, this field is generated using an elongate wire electrode which (i) is electrically isolated from point electrodes located at ports 230a and 232, (ii) enters region 226a via port 232, and (iii) spans the upper edge of region 226a.

Figs. 9 and 10 illustrate another embodiment wherein the first electrophoresis region is bordered by a membrane that segregates this region from an external electrode used in the second electrophoresis step. For example, apparatus 200 from Figs. 6-7 can be modified so that the upper edges of region 226a are bordered by a membrane 290 which defines the upper surface of region 226a. Membrane 290 separates region 226a from an electrode

reservoir 292 that contains an electrode 294 which is linkable to a voltage source 296. The membrane is preferably permeable to small ions but not to the sample components of interest. Thus, the membrane preferably has a molecular weight cutoff (pore size) that is smaller than the smallest sample component of interest. Preferably the molecular weight cutoff is less than or equal to 3000 MW, and more preferably less than or equal to 1000 MW. Any appropriate membrane material can be used, such as a cellulose or cellulose acetate, for example, and many such membranes are available commercially.

For electrophoresis in the first dimension, regions 226a and 226b are filled with appropriate separation media, and electrode reservoir 292 is filled with a sufficient amount of buffer to keep membrane 290 wetted while keeping electrode 294 dry or otherwise electrically insulated from the separation chamber. After electrophoresis in the first dimension is complete (*e.g.*, by imposing an electric field between ports 230a and 232), more buffer is added to reservoir 292 so that electrode 294 is submerged in the buffer. Electrophoresis in the second dimension can then be performed by applying an electric field between electrode 294 and an electrode located at port 235 or between ports 234a and 234b. Conveniently, plates can be manufactured for this embodiment by cutting off the upper ends of plates 220 and 222 using a rotary saw, to produce relatively smooth, flush ends for contacting membrane 290. If desired, gaskets, such as gasket 291 in Fig. 10, can be included between the membrane surfaces and the plate ends and reservoir 292, to help ensure a liquid-tight seal therebetween. A similar membrane/electrode/reservoir structure can be included at the lower end of the second electrophoresis separation region, to provide an external electrode at that end.

Focusing in the second dimension is typically complete in less than 10 minutes in a field of 500 V/cm (5 kV field over 10 cm). A two-dimensional separation can thus be performed well within one hour.

F. Component Detection and Isolation

Separated components can then be detected, localized, and/or quantified in a variety of ways, including photography, confocal fluorescence or epifluorescence scanning, phosphor imaging plates (for radioactivity detection), and CCD (charge-coupled detection), depending on the nature of the signal being detected. According to one advantage of IEF focusing in the second dimension, detection can be performed while the electric field is maintained across the IEF region, to allow integration of signal over time while bands are

maintained in position by the field. Positions of the focused components are quite stable over time, *i.e.* for at least 10 minutes, as long as the field is maintained and no significant turbulence is introduced into the channels.

The components are preferably indexed and recorded using an automated recording device, such as a computer-controlled digital imaging device (*e.g.*, CCD) linked to a low-power microscope. Optical absorbance densitometry techniques can also be used. Since at least one plate is typically optically transparent, to allow for photoactivation of the binding reagent on the removable solid phase, transmission-type signal detection can be used, as in the case of fluorescence detection involving excitation or illumination from beneath, and detection above, the plates. Alternatively, signal generation (if necessary) and detection can take place above a single plate surface, *e.g.*, for fluorescence detection or radiolabel detection. Preferably, fluorescently labeled components are detected via visible light (>450 nm).

Upon imaging the separation and evaluating bands of significance, each site corresponding to a desired component is irradiated at a wavelength effective to photolyze the photocleavable group in the binding compound. As described above, photolysis generates an active group, such as a nitrene, which reacts with the isolated component (*e.g.* a protein) at that site to produce a linkage between the component and the solid phase.

Phenyl azide compounds such as SADP, described above, typically have absorption maxima at around 265-275 nm. Substitution of the phenyl group with electron withdrawing groups shifts absorption to higher wavelengths (Ji and Ji; Jaffe). Benzophenone compounds, described in Oatis *et al.*, also absorb at longer wavelengths. Use of wavelengths >300 nm is more effective when using a glass cover plate, although longer exposure times may be needed, in the case of some substituted phenyl azides (Ji and Ji). If degradation of the photocleavable linkage by ambient radiation during electrophoresis or handling is problematic, the cover plate, whether glass or quartz, can be provided with an opaque cover prior to irradiation, or the separation process can be carried out in reduced light.

Bands are irradiated individually via an optical device of sufficiently narrow diameter to minimize cross-irradiation, *e.g.*, a fiber optic cable. Very high resolution can be achieved, if necessary, by employing masking methods well known in the art,

including photolithographic methods.

Following immobilization of selected components to the solid phase, unbound components are removed by washing, and the solid phase containing only the selected components is then recovered. Washing can be carried out prior to removal of the solid phase from the channels if desired. Flow of the aqueous medium is initiated, by opening appropriate ports in the device, to remove non-immobilized components. Typically, additional wash solution will be introduced through an appropriate port, such as 232 in Figs. 6-11, and passed through the separation channels.

Capillaries connected to the channels can be used for collection if desired. For each selected channel, a capillary tube is inserted into the egress port 236,238, and fluid and/or beads are withdrawn through the capillary by positive pressure, vacuum, piezoelectric pumping, or electroosmotic pumping. Collection may be channel by channel, with or without fractionation, depending upon the number and complexity of selected analytes and the ability of the subsequent analysis (*e.g.*, mass spectrometry) to discriminate individual analytes in mixtures. If desired, a diode detector can be used at the end of the capillary tube to monitor sample bands entering the tube.

If fractionation of a given channel is not necessary, the beads are collected by filtration of the channel contents and washed as a mixture. This method is feasible if, for example, the number of immobilized components is relatively small, their identity can be determined by their location within the second electrophoretic region, and/or they can be easily analyzed as a mixture. In a typical protein differential analysis, for example, up to about 250 isolated components can often be handled in this manner. Beads which are collected without fractionation, or with only partial fractionation, may be sorted by a FACS (fluorescence activated cell sorting) technique, to separate fluorescently labeled beads (*i.e.* those having an attached analyte) from nonlabeled beads.

The components can be released from the solid phase, by chemical cleavage of the second linkage in the binding reagent, and recovered and/or analyzed in solution. As described above, this chemical cleavage may be, for example, reduction of a disulfide or azo linkage or hydrolysis of an ester. The stage of release (*i.e.* after fractionation of the beads, or without fractionation) is again a matter of choice depending on the number of isolated components and how easily they may be separated or analyzed as a mixture.

G. Other Separation Schemes

Other two-dimensional separation embodiments encompassed by the invention include the following first-dimension/second-dimension combinations:

Non-SDS Denaturing/Non-SDS Denaturing. In this embodiment, the separation cavity contains different media in the first and second electrophoresis regions such that the basis of sample migration in the second dimension is different from that of the first dimension (*i.e.*, sample migration in the second dimension depends on sample features different from those in the first dimension). For example, the first separation region can contain a medium with an acidic pH (*e.g.*, 2.5) and a low concentration of sieving components (*e.g.*, 2% linear acrylamide), and the second region can contain a medium with a more basic pH (*e.g.*, 8) and a higher concentration of sieving components (*e.g.*, 4% linear acrylamide).

IEF/SDS-Electrophoresis. In this embodiment, the first electrophoresis region contains an immobilized pKa gradient within region 26a, with an appropriate low-ionic strength buffer, the second electrophoresis region contains an SDS-containing buffer (but not a pKa gradient), and plate 122 additionally contains a lateral slot 190 (not shown) in loading region 160, just above region 26a, which is similar in dimensions to lateral slot 140. After electrophoresis in the first dimension, an SDS-containing buffer is loaded into region 160 via port 135, with egress through slot 190. An electric field is then applied between slot 190 and slot 140 so that SDS molecules diffuse into region 26a. The SDS molecules associate with, and impart negative charge to, the neutrally charged sample components in region 26a, so that the components are drawn into region 26b for size-based separation.

Affinity separations, in which components bind to antibodies or other types of ligands contained in a separation matrix, are also contemplated. It will be appreciated that the invention can be adapted to other combinations of separation modes, according to the needs of the user.

III. Utility

The present invention provides methods and apparatus for characterizing, detecting and/or identifying up to hundreds or thousands of components in a sample on the basis of different charge and mass properties. The invention therefore has utility in a number of applications, including "fingerprinting" samples, *e.g.*, for differential display to facilitate identification and differentiation of samples; detecting and/or monitoring compositions of

normal and diseased cells and tissues; diagnosing or monitoring disease; characterizing or monitoring molecular expression levels of gene products; characterizing the effects of the addition, mutation, deletion or truncation of genes; detecting, identifying, distinguishing, or otherwise characterizing viruses, bacteria, fungi, and other microbes, or components or products thereof; monitoring analyte levels over time as a function of environmental change, life cycle, or exposure to exogenous chemicals or stimuli; toxicity testing; and testing drug candidates for therapeutic efficacy. The method is particularly of interest in studying and characterizing protein components of biological samples, and therefore is useful in proteome research (Wilkens *et al.*, 1997).

From the foregoing, it can be seen how the objectives and features of the invention are met. The invention provides a method that permits two-dimensional electrophoresis and isolation of components in a single apparatus. The apparatus is simple to use and can generate analytical results more rapidly and reproducibly than previous two-dimensional methods. The method permits characterization of samples containing hundreds or thousands of components under a variety of different separation conditions. The use of separation channels containing a removable, functionalized solid phase in the second dimension facilitates collecting selected lanes or individual analytes for further characterization, with retention of resolution, after the pattern of analytes has been imaged and indexed.

Although the invention has been described with respect to particular embodiments and examples for purposes of illustration, it will be appreciated that various modifications can be made without departing from the scope and spirit of the invention.

It is claimed:

1. A two-dimensional electrophoresis system for separating and recovering components within a sample, comprising

(a) an electrophoresis plate assembly which defines a sample separation cavity, bounded by a lower plate and an opposing upper plate, said cavity comprising

(i) a first electrophoresis region containing a first flowable aqueous medium, and adapted to perform charge and/or size-based electrophoresis in a first dimension; and

(ii) a second electrophoresis region, abutting the first electrophoresis region, containing a second flowable aqueous medium in physical communication with said first medium, and adapted to perform electrophoresis in a second dimension, in a direction substantially perpendicular to the first dimension;

and containing a plurality of elongate separation channels defined by said lower plate and substantially perpendicular to said first dimension, containing a removable solid phase effective to bind and immobilize separated components following said second-dimension electrophoresis;

(b) electrode means for generating a first voltage potential across the first electrophoresis region, and

(c) electrode means for generating a second voltage potential across the second electrophoresis region.

2. The system of claim 1, wherein the solid phase comprises a plurality of solid particles within said channels.

3. The system of claim 1, wherein said particles are composed of an uncharged polymer.

4. The system of claim 2, wherein said polymer is crosslinked polystyrene or a poly(alkylmethacrylate).

5. The system of claim 1, wherein the solid phase is derivatized with a binding reagent having an activatable group effective to bind said components to said solid phase upon activation.

6. The system of claim 5, wherein the binding reagent contains a photolabile group which becomes reactive with said components upon photolysis.

7. The system of claim 6, further comprising means for exposing said solid phase to irradiation effective to photolyze the photolabile group.

8. The system of claim 9, where said exposing means is effective to selectively irradiate sites within the second electrophoresis region containing selected separated components.

9. The system of claim 5, wherein the binding reagent further comprises a labile linkage, between the activatable group and the solid phase, which is not labile under the conditions of said activation.

10. The system of claim 9, wherein the labile linkage is chemically cleavable.

11. The system of claim 6, wherein the photolabile group is selected from the group consisting of an azide, a diazo group, and a benzophenone.

12. The system of claim 10, wherein the chemically cleavable linkage is selected from the group consisting of a disulfide linkage, an azo linkage, an ester, a glycol, and a sulfone.

13. The system of claim 1, wherein said media are such that the rates of migration of sample components in the second dimension depend on sample properties that are different from the sample properties that determine sample migration rates in the first dimension.

14. The system of claim 1, wherein the flowable medium occupying the first region includes a plurality of linear polyacrylamide molecules.

15. The system of claim 14, where the second electrophoresis region contains an isoelectric focusing region containing a continuous pK_a gradient immobilized on at least one of said major opposing surfaces, and the flowable medium occupying the second region is a low ionic strength aqueous buffer.

16. A method for separating and recovering components within a sample, comprising:

(a) providing a planar substrate defining a planar sample separation cavity which includes:

(i) a first electrophoresis region, containing a first flowable aqueous medium, and adapted to perform charge and/or size-based electrophoresis in a first dimension, and

(ii) a second electrophoresis region, abutting the first electrophoresis region, containing a second flowable aqueous medium, in physical communication with said first medium, and an immobilized continuous pKa gradient, and adapted to perform isoelectric focusing in a second dimension, in a direction substantially perpendicular to the first dimension,

wherein the substrate further defines, within the second electrophoresis region, a plurality of elongate separation channels substantially perpendicular to said first dimension, containing a removable solid phase effective to immobilize said components following said second-dimension electrophoresis;

(b) applying the sample mixture to the first region,

(c) applying a first voltage potential across the first region, under conditions effective to cause the sample components to migrate across the region, such that different components become separated at least partially on the basis of size,

(d) applying a second voltage potential across the second region, such that the migrated sample components migrate into the second region, in a direction substantially perpendicular to the first dimension, and become separated on the basis of their isoelectric points;

(e) activating selected regions of said solid phase such that sample components become immobilized thereto, in regions of said activating;

(f) removing non-immobilized components, and

(f) recovering the immobilized components.

17. The method of claim 16, wherein said solid phase comprises a plurality of solid particles contained within said channels.

18. The method of claim 16, wherein the solid phase comprises a binding reagent having an activatable group effective to bind the components to said solid phase upon said activating.

19. The method of claim 18, wherein the binding reagent contains a photolabile group which becomes reactive with said components upon photolysis, and said activating comprises irradiating the solid phase with radiation of a wavelength effective to photolyze the group.

20. The method of claim 18, wherein the binding reagent further comprises a labile linkage, between the activatable group and the solid phase, which is not labile under the conditions of said activation.

21. The method of claim 16, wherein said removing of step (f) is accomplished by washing the solid phase.

22. The method of claim 20, wherein said recovering of step (g) comprises cleaving the labile linkage to release said components from the solid phase.

23. The method of claim 19, wherein the photolabile group is selected from the group consisting of an azide, a diazo group, and a benzophenone.

24. The system of claim 10, wherein the chemically cleavable linkage is selected from the group consisting of a disulfide linkage, an azo linkage, an ester, a glycol, and a sulfone.

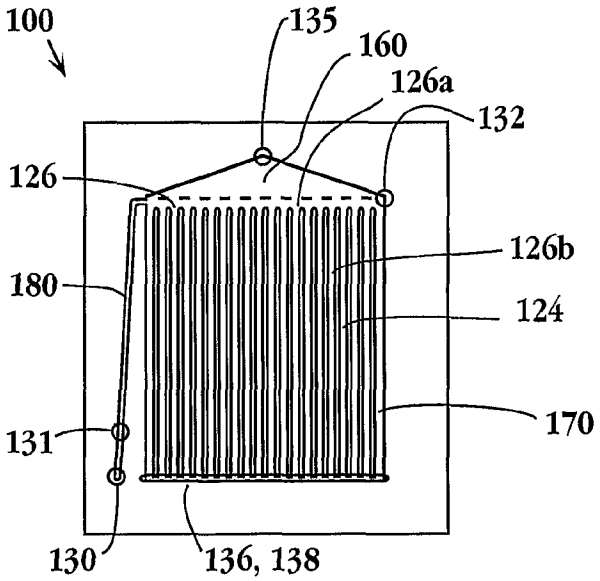


Fig. 1

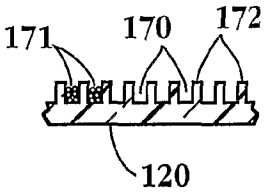


Fig. 3

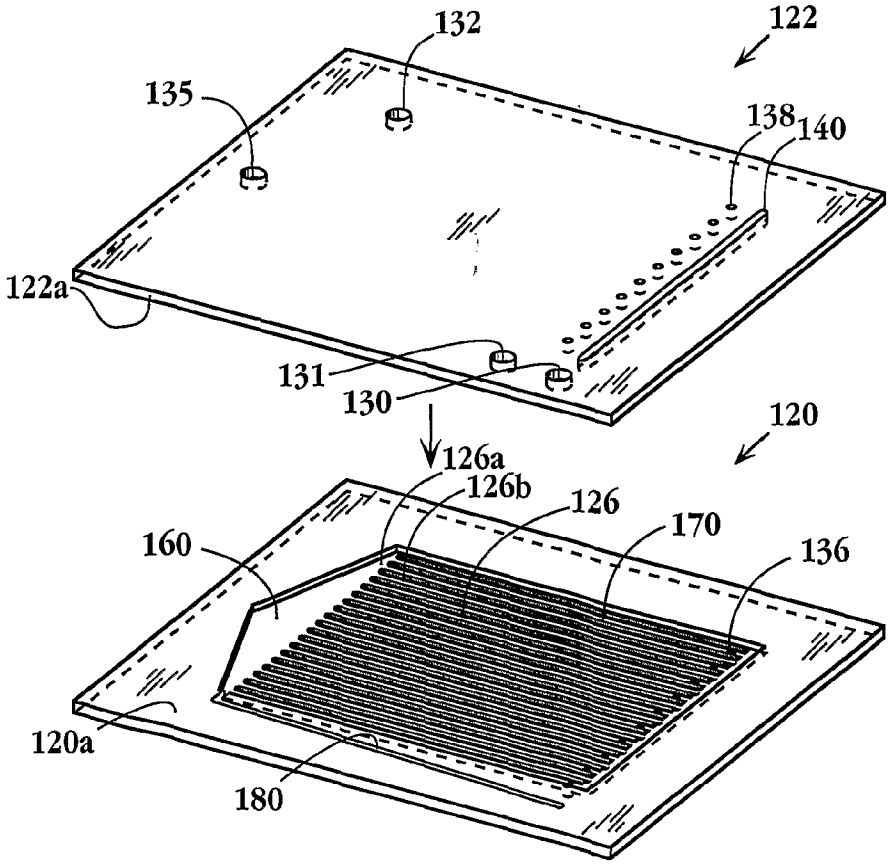
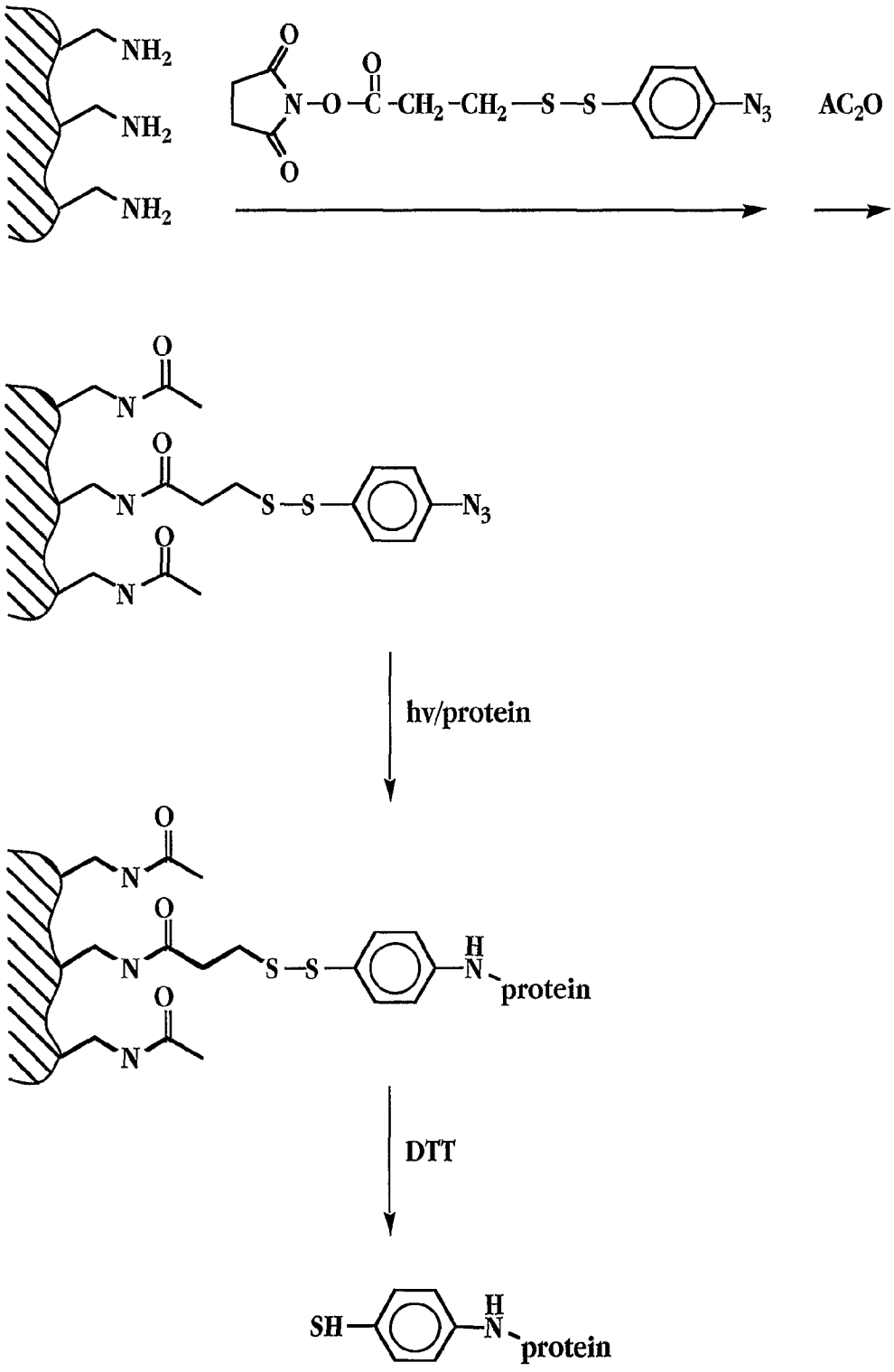


Fig. 2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NH2
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O
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SH
protein

Fig. 4

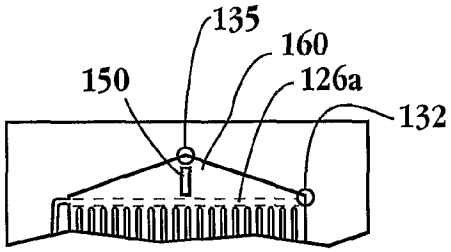

Fig. 5A

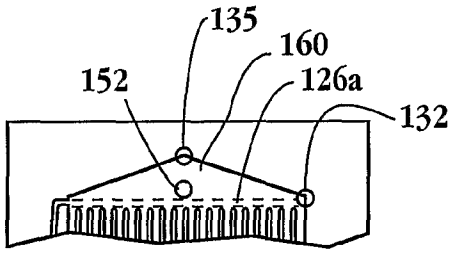

Fig. 5B

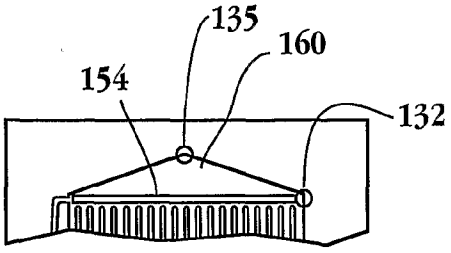

Fig. 5C

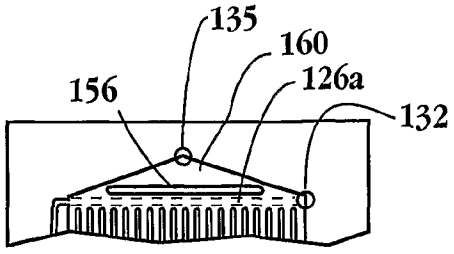

Fig. 5D

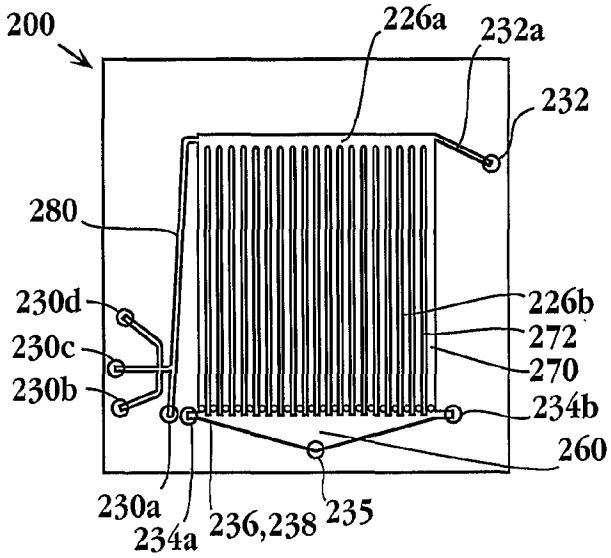
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Fig. 6

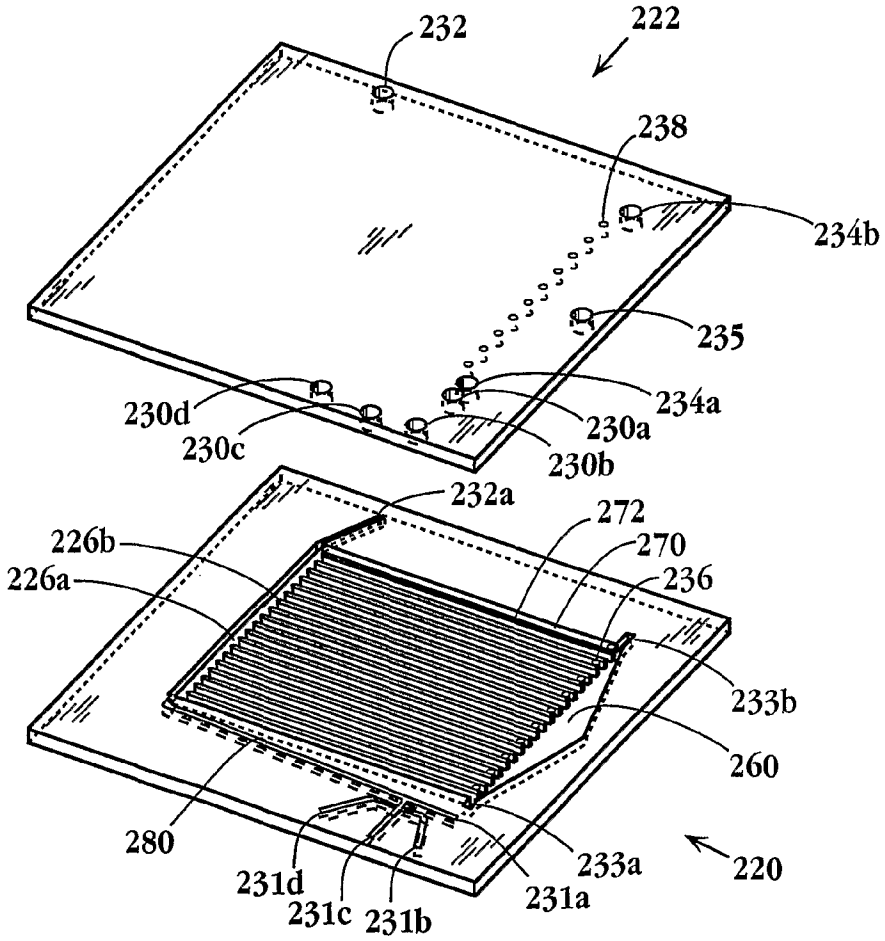
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Fig. 7

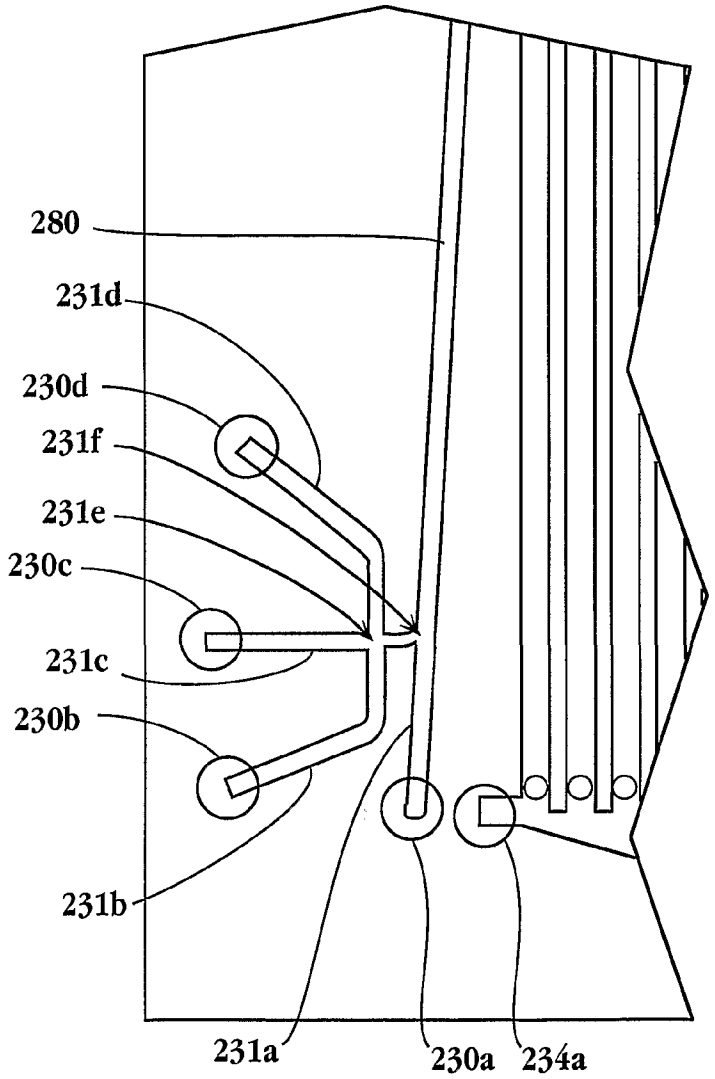
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Fig. 8

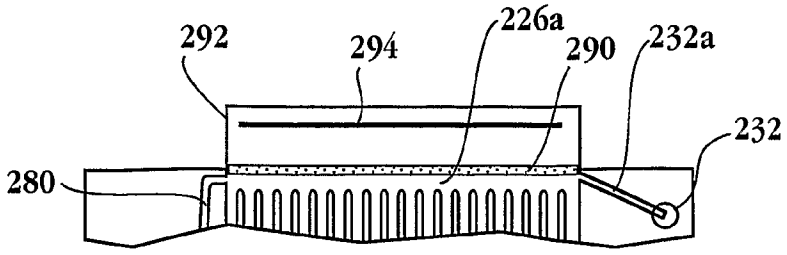
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Fig. 9

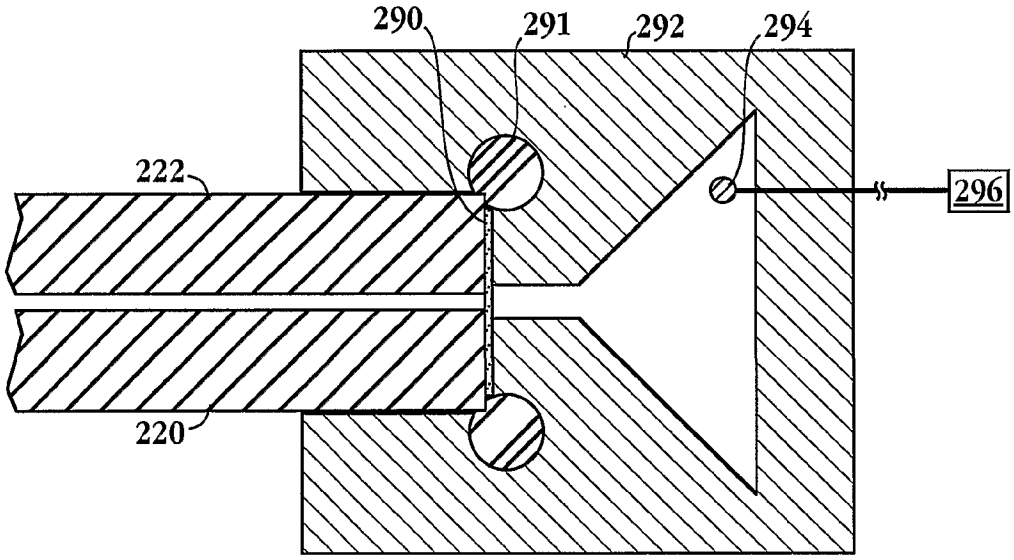

Fig. 10